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EXPERIMENTAL STREPTOCOCCUS EMPYEMA

II. ATTEMPTS AT DYE THERAPY

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In a previous communication, Gay and Stone[1] have described an experimental syndrome in rabbits produced by streptococcus pyogenes and resembling in all essential points human streptococcus empyema. This experimental empyema can be produced invariably under conditions that will be repeated presently and serves as an admirable localized infection on which to work out the possibilities of prevention and therapy. In our previous report we succeeded, though with considerable difficulty and danger to the animal, in producing an active immunity by means of vaccines. Therapy by the use of serum from actively protected animals and containing various immune bodies was successful in only a few instances, although in view of the questionable value of the results obtained by means of vaccine and serum it was judged wise as a next step to investigate the possibilities of chemotherapy with dyestuffs.

Much work has been done concerning the influence of dyestuffs on bacteria and for different purposes, and although a comprehensive review of this subject does not here concern us, it may be serviceable to endeavor to point out the general purposes and results that have been obtained. It was early shown by the work of Rozsahegyi,[2] Stilling,[3] and Koch,[4] that various microorganisms, including the tubercle bacillus, are readily inhibited in their growth (bacteriostasis), or killed by considerable dilutions of dyestuffs, and it was early realized that dye stuffs vary enormously in their bactericidal properties. On the other hand, bacteria were found to vary equally widely in their susceptibility to any particular dyestuff.

The varying susceptibility of bacteria led to the employment of dye stuffs in culture medium for the purpose of isolation and differentiation of certain less susceptible organisms. This was first pointed out by Rozsahegyi[2] and made use of in a practical way in the isolation of typhoid bacillus by Drigalski and Conradi.[5]

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[1] Jour. Infect. Dis., 1920, 26, p. 265.

[2] Centralbl. f. Bakteriol., 1887, 2, p. 418.

[3] Ibid., 1890, 8, p. 155.

[4] Trans. Tenth. Int. Med. Cong., 1890.

[5] Ztschr. f. Hyg. u. Infektionskr., 1902, 39, p. 283.

Much work has been done in an attempt to correlate the chemical structure of dye stuffs with their degree of bactericidal action and, on the other hand, to show the group relationships of bacteria in accordance with their dye susceptibilities. The significance of the latter attempts was focused by the work of Churchman,[6] who showed the definite relationship between the Gram stain and susceptibility to dye action. Subsequently it was indicated that Gram positive micro-organisms are more susceptible than Gram negative bacteria, not only to stains of the para-rosanalin series which are used in this staining method, but also to other groups of dyestuffs as well.

The obvious, practical utilization of these studies, interesting as they are in themselves, lies in the treatment of bacterial infections in the animal body. It may be frankly stated at the outset that we have little assurance at the present time that the interesting results obtained by the action of dyestuffs on bacteria in the test tube may be transferred to the combating of bacterial infections. A number of factors intervene, many of which are clearly understood while others are still in doubt, that interfere with the utilization of dye stuffs therapeutically. It concerns us more particularly to discuss the various factors that intervene and that, in some instances at least, make dye therapy impossible.

Having demonstrated that one of several dye stuffs are bacteriostatic or bactericidal for the kind of mirco-organism under consideration in the test tube, we must obviously first consider whether this particular substance can be used in the animal body in view of its degree of toxicity for animal tissues; in other words, the criterion established by Ehrlich for chemotherapeutic tests must be followed. The substance to be used must be, so far as possible, monotropic, uniting readily with the bacterial protoplasm and not with the protoplasm of the host. The actual physical condition of any kind of dyestuff will be found to influence its toxicity markedly; for example, certain conditions of dilution and heating may render a nontoxic dye colloidal and highly toxic.

Let us suppose that a certain dyestuff is relatively nontoxic for the host and highly bactericidal, we must then assume that it undergoes no essential change until it reaches the bacteria in the body of the host, and we must further be assured that it remains in a condition of simple dilution in the fluids of the animal for a sufficient period of time to assure its complete destructive effect on the bacterial cell. As a matter of fact, a number of things may happen that will prevent these desiderata. The contact between bacteria and dyestuff in the animal body will approximate the test tube conditions only in the case of localized accessible infections, as in wounds or when the dye stuff is injected into closed cavities, as in the particular empyema we are studying. When the dye is given into the general circulation for the purpose of reaching some more or less remote region the results become still further complicated.

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[6] Jour. Exper. Med., 1912, 15, p. 221; 1917, p. 373, 16, p. 822.

In the last and extreme instance when introduced into the circulation many dyestuffs that are tolerated by the animal and highly bactericidal will at first stain the tissues diffusely but after a few minutes or hours the color will be found to have disappeared. On testing various organs or excretions it may be found that the dye has been simply reduced to its leuko base and its color may be restored by oxydation; or the destruction of the dyestuff may proceed further. In both of these instances the bactericidal effect is apparently lost. And again it may be found that although a dyestuff remains generally unchanged in color, it will become rapidly absorbed by certain tissues of the body and may thereby leave the infected areas free from bactericidal action.

A still further limitation to the action of dyestuffs would lie in the possibility of the bacteria concerned becoming dye fast. A thorough consideration of these and other factors concerned in the application of dyestuffs to infections will concern us in connection with the work herewith presented.

THE BACTERICIDAL EFFECT OF CERTAIN DYESTUFFS IN VITRO

We have made a comparative test of a considerable number of dyestuffs in an attempt to find those most active against the streptococcus. Although not primarily interested in the relation of chemical structure to bactericidal properties or the relation of specific dyestuffs to certain bacteria, we have incidentally studied the bactericidal effects of the dyes under investigation, not only on the streptococcus but also on B. typhosus and staphylococcus. Certain general observations may be made from our results which may be of interest apart from the specific action of these dyes on streptococcus.

Considerable differences in the results of various investigators have probably arisen from differences of technic and in many instances the particular technic in bactericidal experiments with dyestuffs has not been given. The technic would vary with the object in view. In our own experiments, dealing as we have with a massive and localized infection, we have utilized large numbers of bacteria in every test. The standard technic on which our results are based consisted in preparing a series of tubes of dyestuff in successive dilutions from 0.1% to the eventually found limit of activity. The dyes were first made up in 1% solution in sterile distilled water and subsequent dilutions made from them by adding beef infusion broth of a P_H of 7.2 to 7.5. With all the active bactericidal dyes, and indeed, with most of the others, such dilutions were found to be perfectly sterile. In a few instances dyes were autoclaved after dilution, with variable results as regards their bactericidal properties. To a series of dilutions was added an equal amount (1 c c) of 24-hour broth culture of streptococcus "H" or the other organisms to be mentioned. The tubes were then incubated for 24 hours and streaks on blood-agar plates were made to test viability. In most instances this arbitrary limit of 24 hours was found to give the ultimate degree of bactericidal property, although in some cases further incubation for another 24 hours led to sterility in a higher dilution. In a number of instances it was found, moreover, that the limit of bactericidal property was the same irrespective of the number of organisms introduced, at least within the limits of from 1 c c as ordinarily used to 0.1 c c of the same culture.

A brief statement of the bactericidal properties of the dyestuffs we have tested in respect to the various micro-organisms under consideration follows:

1. The following dyes[7] were not bactericidal for streptococcus "H," for staphylococcus aureus 112, or for B. typhosus 3 in a final dilution of 1:2000:

Benzo-azurin (H)
Azo-acid red (N)
Diamil blue (J)
Erioglaucin A. (G)
Acid fuchsin (J)
Congo red (J)
Cyanin B. (M)
Oxamin violet (B)
Sauer grün (J)
Scarlet 6 R. (M)

2. Acid violet, although inactive, at the dilution of 1:2000, for B. typhosus and streptococcus was found bactericidal for staphylococcus at 1:20,000.

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[7] We are indebted to Prof. H. M. Evans for many of the dyes employed and for information concerning them. The dyes were from the following manufacturers as indicated by the letter following the name:

A., Berlin Anilin Works.
B., Badische Co.
By., Bayer Co.
C., Cassella.
D., A. H. Thomas Co.
E., Eimer and Ahmend Co.
F., Hynson, Westcott and Dunning.
G., Geizy.
H., Harmer Lab. Co.
I., Boots Co.
J., Grübler.
L., A. Leonhardt & Co.
M., Meister, Lucius and Bruening, Ltd.
N., H. A. Metz.
O., Elberfeld.
P., Society of Chemical Industry, Basle.

3. The following dyes were found bactericidal for streptococcus, but not for staphylococcus and B. typhosus at a dilution of 1:2000:

Acridine orange (L)
Brilliant cresyl blue (H)
Columbia blue R. (A)
Crystal ponceau (A)
Methylene blue (med. pure) (J)
Methylene blue (G. G.) (C)
Nile blue (By)
Neutral red (J)
Safranin (H)
Sulphon acid blue R. (O)
Wasser blau (B)
Toluidin blue (J)
Janus dark blue B (M)

4. The following dyes were found bactericidal for streptococcus and staphylococcus but not for B. typhosus at a dilution of 1:2000 or more:

Basic fuchsin (J)
New fast green 3 B. (P)
Rhodulin violet (By)
Setocyanin (G)

5. The following dyes were found bactericidal for streptococcus, B. typhosus and for the most part for staphylococcus at dilutions of 1:2000 or more, as expressed comparatively in table 1.

TABLE 1

DYES BACTERICIDAL FOR STREPTOCOCCUS, STAPHYLOCOCCUS AND B. TYPHOSUS

	Streptococcus	Staphylococcus	B. Typhosus
Auramine (E)	2,000	0	2,000
Methylene green (B)	2,000	0	2,000
Acriflavine (F)	20,000	20,000	20,000
Janus black "2" (M)	20,000	0	2,000
Crystal violet (A)	200,000	20,000	20,000
Proflavine (I)	200,000	20,000	20,000
Spiller's purple (J)	200,000	20,000	2,000
Janus green (M)	200,000	2,000	2,000
Brilliant green (D)	2,000,000	20,000	2,000
Malachite green (A)	2,000,000	20,000	2,000
Methyl violet (H)	2,000,000	20,000	2,000
Solid grün (J)	2,000,000	20,000	2,000

Some general conclusions would seem warranted from the results obtained. In a general way both streptococcus and staphylococcus are more susceptible to dyestuffs, with few exceptions, than is B. typhosus. Generally speaking, the streptococcus studied is more susceptible than the strain of staphylococcus. These suggestions may possibly be offered: It would seem that diazo dyes have relatively little bactericidal properties. The most active dyes for streptococcus belong to the triphenylmethane and acridine group.

At this point may be mentioned certain other test tube experiments we have undertaken as bearing on certain limitations of dye action. It would be suggested from the work on the chemotherapy of protozoan infections that an inefficiency of dyes on bacteria might exist if the latter were able to acquire gradually "dye fastness." Browning and Gulbransen[8] have actually tested for fastness with flavine and brilliant green and found no indication of its existence. Simon and Wood,[9] however, have claimed that dye fastness may arise. Although our experiments are not extensive on this point, they are unequivocal and show clearly that repeated subcultures of the streptococcus in the highest nonbactericidal dilution of several of the most active dyes (acriflavine, new fast green 3 B, safranine and malachite green) not only did not render the micro-organisms more resistant to more concentrated solutions of the dye, but also actually seem to develop a greater susceptibility to the dye in dilutions in which it originally grew.

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[8] British Med. Jour., 1917, 1, p. 73.

Several experiments were undertaken to consider the possibilities of the mixture of bactericidal dyes. It was thought possible that a combination of dyes might be more bactericidal than either of them alone. It was found, however, that the final bactericidal dilution of such mixed dye solutions might reach the limit of the stronger dye, but in case of interaction and precipitation between the two solutions the bactericidal effect was actually decreased.

As we have already mentioned, one of the recent important advances in the knowledge of the action of dyestuffs on bacteria has been the appreciation that many of them, although active under conditions of artificial cultivation of bacteria in the test tube, are inactive in protein solutions that simulate conditions in the body. The recent advocacy of the use of the flavine or acridine compounds in the treatment of wounds lies in the demonstration by Browning and Gulbranson,[8] Hewlett,[10] Dakin and Dunham [11] and others, that in contradistinction to most dyes these compounds are fully as active or even more active against bacteria in protein solutions than in broth or salt solution. We will take up in connection with the experiments in the living body, the other advantages that are claimed for them, but it was obvious that before proceeding to animal experiments we should test out the various dyestuffs that have been found to be most active in broth solutions against the streptococcus on this micro-organism as it actually occurs in our experimental empyema.

The experimental empyema in rabbits is produced by injecting 0.2 c c of a 24-hour broth culture of a passage strain of Streptococcus pyogenes "H" into the right pleural cavity. The passage strain is conserved in the pleural fluid derived from previous cases which may be kept in the icebox without deterioration for several weeks. Although such pleural fluids become sterile at room or incubator temperature in a few days, the number of streptococci increases gradually in the icebox for about 3 weeks and then gradually decreases although subcultures are positive for at least 2 months. It should further be noted that although the samples of fluid are taken from the infected pleura at necropsy with only moderate precautions and placed in large tubes that are repeatedly opened for subcultures, they have never been found contaminated. This passage culture has assumed fixed virulence so that it succeeds invariably in producing a progressive pleuritis extending from the right side to the left through the pericardium without producing septicemia except in the terminal stages.[12] Infection is fatal in from 3 to 7 days at which period 20 c c to 30 c c of a purulent exudate is found in each pleural cavity.

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[9] Amer. Jour. Med. Sci., 1914, 147, p. 524.

[10] Lancet, 1917, 193, p. 727.

[11] Handbook of Antiseptics, 1918.

A series of tests have been made with the dyestuffs that were found active in broth against the streptococcus in dilutions of 1:2000 or better on this pleural fluid derived from fatally infected rabbits. The technic of these experiments was similar to that described for the broth cultures and in each instance 1 c c of pleural fluid was added to the dye dilution. Several estimates of such fluids show that a cubic centimeter contains from 200 million to two billion streptococci as compared with a 24-hour broth culture which on an average contains one billion organisms. A comparative action of the various dye stuffs as against streptococci broth cultures and in pleural fluid is shown in table 2.

TABLE 2

THE BACTERICIDAL TITER OF VARIOUS DYESTUFFS ON S. PYOGENES "H" IN:

	Pus	Broth Culture
Buffalo fast crimson R (?)	0	2,000
Columbia blue R (A)	0	2,000
Crystal ponceau (A)	0	2,000
Methylene green (B)	0	2,000
Nile blue (By)	0	2,000
Wasser blau (B)	0	2,000
Sulphon acid blue (?)	0	20.000
Spiller's purple (J)	0	200,000
Setocyanin (G)	0	200,000
Pyronin (E)	0	200,000
Brilliant cresyl blue (H)	2,000	20,000
Methylene blue G. G. (O)	2,000	20,000
Methylene blue (Med. Pur.) (J)	2,000	200,000
Janus black "2" (M)	2,000	20,000
Janus dark blue (M)	2.000	200,000
Neutral red (J)	2,000	200,000
Toluidine blue (J)	2,000	200,000
Brilliant green (D)	2,000	2,000,000
Malachite green (A)	2,000	2,000,000
Methyl violet (H)	2,000	2,000,000
Safranin (H)	2.000	2,000
Methylene green (B)	2,000	2,000
Acridine orange (L)	20,000	200,000
Janus green (M)	20,000	200,000
New fast green 3 B (P)	20,000	200,000
Basic fuchsin (J)	20,000	20,000
Rhodulin violet (By)	20,000	20,000
Solid grün (J)	200,000	2,000,000
Acriflavine (F)	200.000	20,000
Proflavine (I)	200,000	200,000

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[12] Twenty-seven normal controls have been positive; of 85 other animals treated in various ways in an attempt to modify conditions only 2 have survived, and these were treated with an immune serum. There may be contrasted with these figures, actively immunized animals which, as shown in the previous report, are fully protected.

Most of the dyes studied lose markedly in bacterial power in the presence of pus. A considerable number remain rather active and a few of them, safranin, methylene green, basic fuchsin, rhoduline violet and the two flavine compounds are as active in pus as in broth. Acriflavine, as in Browning's[6] experiments with serum, is actually more active in pus than in broth.

THE ACTION OF SELECTED DYES ON EXPERIMENTAL EMPYEMA

We have carried out series of experiments on experimental empyema in rabbits with certain of the dyestuffs chosen from among those that were found active in vitro on streptococcus pus from this condition. With 5 of these dyes—janus green, solid green and safranin, methylene blue (med. pure) and new fast green 3 B—our observations were merely casual and were not continued because no encouraging results were obtained. These experiments may be briefly summarized:

1. *Exper. with Safranin.*—Rabbit 17, weight 1,500, Feb. 16, 1920, was inoculated in the right pleural cavity with 0.2 c c of a 24-hour broth culture of S. pyogenes "H" from pleural fluid (such injection which is invariably positive as already noted will hereafter be referred to simply as infection with streptococcus).

On the following day there was injected into the same pleural cavity 5 c c of a 0.5% solution of safranin in normal saline. The animal was found dead on the following day and at necropsy the entire musculature of the thorax showed a faint pink color and the diaphragm was a deep pink color. The urine was distinctly colored. The right pleural cavity contained 26 c c, slightly cloudy, highly colored fluid, containing grumous masses. The left pleural cavity contained 6 c c of the same turbid fluid. Cultures from both cavities were positive for S. pyogenes. In this experiment it should be noted that apart from the possible toxicity of the dye for the animal, the dye remained to a large extent localized but did not sterilize. Judging from the amount of fluid obtained in the right pleural cavity there must have been for a period of 24 hours a dilution of the dye stuff of approximately 1:1,000 which may be compared with the bactericidal titer of this dye in pus, of 1:2,000.

2. *Exper. with Janus Green.*—Rabbit 46, weight 1,250, was infected on May 11 with streptococcus and on May 12 injected into each pleural cavity 1 c c of a 0.1% solution of janus green B. The animal was found dead on the following day and at necropsy presented these findings: The right pleural cavity contained 12 c c of fibrinopurulent pus of a light magenta shade. (It should be noted that janus green is a derivative of safranine, according to Green[13]). The left cavity contained fluid of the same shade. The organs adjacent to the diaphragm were all stained with the same magenta color. Cultures from both right and left cavity were positive for streptococcus.

In this experiment, although the dyestuff was apparently reduced, it should be noted that the dilution of the dye in the right cavity was approximately

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[13] Organic Coloring Matters, 1908.

1: 1,200 and in left cavity 1: 400, whereas the bactericidal titer of this dye in pus had been found to be 1: 2,000.

3. *Exper. with Solid Green.*—Rabbit 48, weight 1,200, was infected May 11, 1920. On the following day the animal received 1 c c of 0.1% solution of solid green in each pleural cavity. It was found dead on the following day and at necropsy the right cavity contained 3 c c of fibrinopurulent pus of light green color. The lung was apparently in good condition. The left cavity contained 1.5 c c of clear green fluid. The base of the left lung was consolidated. The cortex of the kidneys was stained green. The urine was normal in appearance. Cultures from the right pleural cavity were positive; cultures from the left, which was the last to be invaded, proved sterile. In this cavity the concentration of dyestuff equals 1: 1,500. In the right cavity destruction of the organism was not accomplished in dilution of 1: 3,000. The bactericidal titer in vitro of this dye in pus proved to be 1: 200,000. No apparent disintegration of the dye had taken place.

Rabbit 53, weight 1,500, was infected May 18, 1920, and 6 hours later it received 1 c c of 0.5% solid green solution in each pleural cavity. The animal was found dead the following day, and the right cavity contained 5 c c of green pus with a small amount of fibrin. Pericarditis was present. The left pleural cavity contained 2 c c of bloody fluid. Cultures of both cavities were positive. In this case the presumed dilution of dye was 1: 1,000.

4. *Exper. with Methylene Blue.* — Rabbit 317, weight 2,500, was infected April 20, 1920, and the following day received 2 c c of a 1% solution of methylene blue (med. pure Grübler) in each cavity. The animal was found dead the following day. The hair about the external genitals and the urine was found stained a bright blue. Six c c of bright blue pus was found in the right pleural cavity. The lung was congested and covered with a fibrinous exudate. The left cavity contained 6 c c of a turbid slate colored fluid. Cultures from both cavities were positive for streptococcus. The dilution of dye in this cavity was 1: 300, whereas the bactericidal pus titer as tested in vitro was 1: 2,000.

5. *Exper. with New Fast Green 3 B.*—Rabbit 40, weight 1,900, was infected April 6, 1920. On the following day, it was given 2 c c of the supernatant fluid of a 0.5% solution and suspension of new fast green 3 B in sterile salt solution at room temperature.[14] Twenty-four hours later the right pleural cavity was aspirated and 9 c c of pus of a brownish color removed. Positive cultures were obtained from this pus. On the next day, that is, 4 days after infection, the animal was found dead. The right pleural cavity contained 4 c c of bloody turbid fluid, the left cavity 5.5 c c of the same. No green coloration was evident except about the inferior vena cava and the musculature and cartilage of the trachea. Positive cultures from both cavities were obtained. In this instance the original dilution of dye, which apparently lost its color in pus, was in a concentration of 1: 1,000, whereas the bactericidal titer outside the body had been found to be 1: 20,000.

Experiments with Acriflavine.—Our experiments with the acridine compounds, particularly the one known as acriflavine, are more extensive and would seem to have exhausted the logical possibilities for the use of this dye in treatment of experimental streptococcus empyema. As we have already indicated, work in recent years has tended to bring these dyestuffs into prominence because they have been found not only highly bactericidal in broth and salt solution, but also in protein mixtures (Browning [6] and Hewlett [10]). Our own experiments and those of Dakin and Dunham [11] have confirmed these observations. It is further asserted by Browning and his collaborators that acriflavine does not inhibit phagocytosis, that it penetrates tissues, and gives rise to no "dye fastness" in bacteria. Our experiments in the living animal have been carried out entirely with acriflavine, owing to the fact that it is somewhat more bactericidal in pus solutions than proflavine as judged from our own work and from that of Dakin and Dunham. Browning and Gulbransen,[15] moreover, have tested these substances in mice and found acriflavine somewhat less toxic than proflavine, although it was judged that both could be used effectively in tolerable doses. Although Browning and Gulbransen found that pneumococcus infections in mice could be inhibited by acriflavine, living organisms still remained in certain tissues. The actual results obtained from the use of these substances in infections we shall refer to more fully in commenting on our own results. The only objection that has been raised to the possible effectiveness of acriflavine compounds is that they are somewhat slow in action. It is indicated from the work of Browning and his collaborators and from our own work also on acriflavine, that it does not become changed in the body although it has an elective affinity for muscle. The following examples are chosen from an extensive series of animals as illustrative of the successive phases through which our work has passed and the characteristic results obtained.

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[14] It should be stated here that new fast green 3B is completely soluble in water or salt solution in about 1/16 of 1%. In efforts to dissolve larger amounts of this dye, higher temperatures have been resorted to with the result that although the dye gives perfect solution while warm and may subsequently remain fluid at body temperature, it jells on cooling. Coincidently with this colloidal suspension of the dye, a striking increase in its toxicit appears as well as its ability to stain the animal body diffusely. Under the conditions mentioned in this protocol the dye is apparently not toxic and does not stain diffusely.

6. *Exper. Illustrating the Tolerance of the Normal Rabbit for Acriflavine.*—Rabbit 13, weight 1,300, received 5 c c of a 0.1% solution of acriflavine in sterile salt solution in the right pleural cavity. There were no symptoms. The dose was repeated on the next day and on the following day the animal was killed and examined. The intestine and stomach were not colored, the urine was stained a deep yellow. The walls and musculature of the right pleural cavity were stained faintly yellow, as was also a persistent thymus. A small amount of clear fluid was found in the left pleural cavity. The right lung had a few spots of atelectasis, otherwise nothing abnormal was noted.

7. *Exper. Showing Action of Acriflavine in Empyema.*—Rabbit B, weight 2,750, was infected with streptococcus pyogenes on Jan. 30, 1920. Three days

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[15] Jour. Hygiene, 1919, 18, p. 33; Jour. Path. and Bacteriol., 1919, 22, p. 256.

later it received 5 c c of a 0.5% solution of acriflavine, warmed to body temperature, in the same pleural cavity. On the following day the animal was exsanguinated and necropsy performed. There was no coloring of the abdominal viscera. The pectoral muscles were stained yellow. The right pleural cavity contained 33 c c of light straw colored fluid, which was much less turbid than in untreated animals. Masses of purulent fibrin were found on both visceral and parietal pleura. The left cavity contained 9 c c of a more turbid but clear yellow fluid. There was a marked fibrinous pericarditis. Smears from the pleural fluid showed many phagocytes with streptococci and with no extracellular organisms. Cultures from both left and right cavities were positive.

It should be noted in this instance that the concentration of acriflavine in the right pleural cavity was approximately 1:1,200, whereas the bactericidal titer of this dye in pus had been found to be 1:200,000. Twenty-four hours later, as found at necropsy, the fluid in the right pleural cavity was tested and found to resemble in color a dilution of acriflavine of from 1:10,000. In spite of these facts, the cavity had not become sterilized. It seemed possible that the dye had been given too late to be effective.

Exper. 8.—Rabbit A, weight 2,650, was infected as usual on Jan. 30, 1920, and on the following day given 5 c c of 1% acriflavine in sterile salt solution in the same pleural cavity. The animal showed symptoms of irritation after injection. Death occurred on the following day, and at necropsy the following findings were obtained. The muscles of the thorax and abdomen were stained yellow. There was considerable turbid peritoneal fluid. The right pleural cavity contained 20 c c of reddish brown turbid fluid. The right lung was consolidated at the base and showed adhesions to the thoracic wall. The left pleural cavity contained 10 c c of brightly colored fluid. Cultures from the right pleural cavity were positive in spite of the fact that the dilution of acriflavine must have approximated 1:400. The left pleural cavity was sterile, which may have been due to the failure of the infection to extend to this point, but which suggested that an actual sterilization had occurred.

Exper. 9.—Rabbit 1, weight 2,400, was infected on Feb. 4. The treatment differed from preceding experiments in that 5 c c of the 0.5% of acriflavine solution were given 24 and again 48 hours after inoculation. The animal was found dead 3 days later and at necropsy showed an extensive double pleurisy with 25 c c of fluid in each pleural cavity, which had lost any distinctive yellow color. Cultures from both pleural cavities were positive.

It was evident in this experiment that whatever may have been the temporary action of two relatively large doses of acriflavine, the result was unsuccessful so far as ultimate sterilization was concerned. The animal, moreover, apparently did not die of the action of acriflavine but showed the normal course of an untreated infection, namely, 5 days.

Exper. 10.—Rabbit 3, weight 2,300, was infected as usual and given a single dose of 5 c c of 0.5% acriflavine. The animal was found dead 3 days later and at necropsy 25 to 30 c c of yellow fluid was found in each pleural cavity, which was very clear and not as turbid as in other animals. Cultures taken immediately from this fluid were positive, but when cultures were made 2 days later from the same fluid preserved in the icebox, it was found that the right pleural fluid gave a negative culture and the left showed only a few organisms.

This experiment suggested the possibility that the failure to sterilize empyema cavities in rabbits might be due to a failure of the dyestuff to work in cavities relatively free from oxygen, as the fluids outside the body in contact with the air became sterile. It should be reiterated that the normal fluids remain viable for many days. This result was obtained in several other experiments. In order to settle the question, dilutions of acriflavine in broth of from 1 : 1,000 to 1 : 1,000,000 were made in a series of Hall's[16] anaerobic tubes with marble device. It should be stated that autoclaving has no effect on the bactericidal effect of acriflavine. These various dilutions were inoculated immediately on cooling both above and below the marble with 0.5 c c of a plain broth culture of streptococcus pyogenes "H," that is to say, they were inoculated both anaerobically and aerobically. The cultures were observed for 3 days and streaked on blood-agar plates finally from the portions above and below the marble. In both instances the cultures at 1 : 100,000 were sterile, whereas those at 1 : 1,000,000 were not. In other words, the dyestuff apparently is as effective in the absence of oxygen, and such absence of oxygen would not account for the failure of the dye to work as expected in the pleural cavity.

Exper. 11.—Rabbit 11, weight 1,800, was infected Feb. 10. On Feb. 12 it received 5 c c 0.5% acriflavine and on the following day 2 c c 0.5% acriflavine in the right pleural cavity. The animal was found dead on the following day. Kidneys and urine were highly colored. The right pleural cavity contained 6 c c of bloody fluid. The lung was consolidated. There was no pericarditis. Little fluid appeared in the left pleural cavity; the apex of that lung was also consolidated. Cultures from the right pleural fluid showed only 3 colonies of streptococcus and from the left the fluid was sterile.

This experiment illustrates that although the acriflavine was pushed to the point of almost completely sterilizing the cavities, recovery did not take place.

Exper. 12.—Rabbit 51, weight 2,200, was infected on May 18 as usual, and on the afternoon of the same day it received 1 c c of a 0.5% solution of acriflavine in each pleural cavity. On the following day both cavities were aspirated 23 c c of straw colored fluid were taken from the right pleural cavity and 22.5 c c from the left. Cultures from both were sterile. The dose of acriflavine was repeated after aspiration and the following day aspiration was again practiced with a removal of 15 c c from each cavity. A culture from the right cavity was negative, from the left positive. The animal died the following day and at necropsy showed slight infection about the point of inoculation. There were adhesions and advance fibrinopurulent exudate in both cavities with about 8 c c of fluid in each. Cultures from both fluids were positive. All other organs appeared normal.

This experiment illustrates the termination of what seemed to us the logical possibilities of utilizing acriflavine alone in sterilizing rabbits suffering from experimental empyema. Not only were doses given resulting in a greater concentration of the dyestuff in the pleural fluid than was theoretically necessary to sterilize the pus present within 24 hours, but it was shown that the dye was not decomposed in the body during this period of time although it had been absorbed to some extent by other tissues, particularly by the muscles. As testing further possibilities, the injections of dyestuff were repeated and in order to remove mechanically large numbers of organisms and relieve respiratory distress, aspiration was practiced several times. Although we have apparently nearly sterilized the cavity, regrowth of the organism has always occurred. In no instance has the life of the animal been prolonged even with changes in the culture findings. The thought naturally arises that the dyestuff does not penetrate sufficiently into the exudate, which has invariably been found on the pleural surfaces, to kill all bacteria. Every distinct change has been noted in the character of the pleural fluids. They become clearer under the action of acriflavine, the masses of fibrin become smaller and more compact. The fact that these exudates are less turbid suggests that the normal activity of the leukocytes, which may to some extent combat the infection, may have been inhibited. The next step in our investigation was to determine whether this had probably occurred.

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[16] Univ. Calif. Pub. Path., 1915, 2, p. 147.

THE EFFECT OF ACRIFLAVINE ON PHAGOCYTOSIS AND THE ADJUVANT ACTION OF IMMUNE SERUM

Browning[8] has asserted that acriflavine has no inhibiting effect on phagocytosis. It seemed important to investigate this point with the conditions under which we were working and in addition to consider the possibility of employing acriflavine in conjunction with immune serum, which had already been shown to have distinct, although slight effect on our experimental empyema. At our suggestion, Mr. D. D. Stafford undertook a series of experiments which, although largely negative for the point under consideration, in themselves are interesting.

Exper. 13. The Effect of Acriflavine on Phagocytosis and in Sterilizing Cultures in the Presence of Leukocytes.—The essentials of the opsonic technic with streptococcus have already been described in our previous report. Leukocytes were obtained by injecting sterile broth into the peritoneal cavity of the guinea-pig. Streptococcus antiserum had been provided by immunizing rabbits whose active immunity was tested by an intrapleural injection. This serum was inactivated by heating to 56 degrees for ½ hour before employment. The organisms consisted of small amounts of 24-hour broth culture of S. pyogenes "H." At the end of 2 hours the tubes were centrifugalized, the supernatant fluid decanted, smears made of the sediment, and opsonic determinations made by counting the percentage of 100 leukocytes that included bacteria. The tubes were continued at 37 C. and streaks made on blood-agar plates to determine the destruction of bacteria at the end of 24 hours. The results of such a test are summarized in the following table.

TABLE 3

RESULTS OF EXPER. 13

Tube	Leukocytes	Immune Serum 1012, 56 C.	Strepto-cocci	Acriflavine	Phago-cytosis in 2 Hours, Percentage	Culture After 24 Hours
1	0.2 c c	0.2 c c	0.2 c c	0.2 c c 1:100	16	—*
2	0.2 c c	0.2 c c	0.2 c c	0.2 c c 200	17	—
3	0.2 c c	0.2 c c	0.2 c c	0.2 c c 400	26	—
4	0.2 c c	0.2 c c	0.2 c c	0.2 c c 800	30	—
5	0.2 c c	0.2 c c	0.2 c c	0.2 c c 1600	45	—
6	0.2 c c	0.2 c c	0.2 c c	0.2 c c 3200	44	—
7	0.2 c c	0.2 c c	0.2 c c	0.2 c c 32000	50	—
8	0.2 c c	0.2 c c	0.2 c c	0.2 c c 320000	44	++
9	0.2 c c	0.2 c c	0.2 c c	0.2 c c Saline	42	++
10	0.2 c c	Saline	0.2 c c	0.2 c c Saline	22	++

* The sign (—) means sterile.

This table brings out several interesting points. Although 22% of the leukocytes contain micro-organisms in the control without serum, nearly 50% include them in the tubes in which serum is present. In the more marked concentrations of acriflavine, that is, from 1:800 to 1:100, a distinction of inhibition of phagocytosis has occurred in increasing grade, whereas there is apparently no inhibition of phagocytes in tubes 5 to 8, which actually contain dilutions of acriflavine of 1:6,400 upward. It is of further interest that in the tubes containing serum and leukocytes alone the bacteria, although phagocyted, are not destroyed, whereas up to an actual dilution of 1:128,000 in tube 7, containing sufficient amounts of acriflavine, destruction has occurred. There is a destructive zone, in tubes 5 to 7, in which the acriflavine has apparently not inhibited phagocytosis and has also sterilized the mixture.

Exper. 14.—With the result of the last experiment in mind, several experiments were conducted to determine whether it could be demonstrated that a greater destruction of bacteria in the presence of leukocytes and immune serum with marked phagocytosis occurs than with leukocytes in normal rabbit serum when less opsonic action is evident. Cultures, furthermore, were made both at 24 and 48 hours in such experiments and there was no demonstrable difference in the sterilization titer of acriflavine whether in immune serum, normal serum or salt and irrespective of the presence or absence of leukocytes. In further experiments alexin from the guinea-pig was added for the purpose of possible reactivation of the immune serum. It had, however, no effect on the results as already determined.

There would seem, therefore, little evidence from experiments in the test tube to suppose that combinations of immune serum and acriflavine would work any more satisfactorily in experimental empyema than either one of them alone. In view, however, of the notorious divergence of results obtained in the test tube and the animal body it was judged to test out these conditions in the infection itself, which led to such experiments as are herewith detailed.

Exper. 15.—Rabbit 22, weight 1,700, was infected on March 17, 1920, and on the following day received into the same pleural cavity a mixture composed of 2 c c rabbit antistreptococcus serum 1012 + 0.2 c c of 1% acriflavine. On the following day 10 c c of brownish gray, cloudy fluid was removed and the treatment repeated. Culture from this fluid was positive for streptococcus. On the following day, that is, 3 days after infection, 5 c c of fluid were removed from the right cavity and 2 c c from the left. Cultures from both were positive. The animal died 2 days later with the following findings: The right pleural cavity contained 3 c c of cloudy bloody fluid; the pleural surface was covered with a dirty yellow exudate. About 10 c c of fluid were present in the left pleural cavity with the same amount of exudate. There was slight pericarditis. Cultures from both pleural fluids were positive.

Exper. 16.—Rabbit 28, weight 1,750, was infected on March 24, 1920. On the following day 1 c c of pooled immune rabbit serum containing 0.125% of acriflavine[17] was injected. The animal was found dead on the following day with characteristic findings in both pleural cavities and with positive cultures from their fluid.

DISCUSSION AND CONCLUSIONS

As already stated, there is little certainty of the ultimate usefulness of dyestuffs as disinfectants in actual bacterial infections. This is true not only of generalized infections, in which case dyes would be given into the circulation, but also in the case of localized infections in which their usefulness might seem more probable. In respect to generalized infections, a few references have been given as to theoretical possibilities; Churchman and Herz[18] have stated that the blood of animals that have been injected with considerable amounts of gentian violet retain an increased bactericidal property for about 2 hours. It is known, however, that these dyestuffs are reduced relatively rapidly and are highly toxic. Crystal violet has actually been given intravenously in the treatment of coccidiodal granulomo by Brown and Cummins[19] and by Cummins and Sanders[20] without definite ill effects but with no distinctive results. In a similar way it has been shown by Browning and Gulbransen[21] that acriflavine endowed the serum of experimental animals with temporary bactericidal properties, and the use of it has been suggested in the treatment of trench fever with results that are claimed to be favorable (Byam, Dimond, Sorapure, Wilson).[22]

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[17] It had been found in the meantime that greater concentrations of acriflavine than this amount gave a distinct precipitate in the presence of serum.

[18] Jour. Exper. Med., 1913, 18, p. 579.

[19] Arch. Int. Med., 1915, 15, p. 608.

[20] Jour. Med. Res., 1916, 35, p. 243.

[21] Proc. Roy. Soc., 1918, 90, p. 136.

The use of dyestuffs in the treatment of wounds and on infected surfaces has been more extensive and was suggested in the earliest work of Stilling,[3] who spoke of their possibilities in the treatment of eye infections. Gentian violet has been employed in wounds infected with diphtheria bacillus by Churchman [23] with apparently good results. Brilliant green has been tried by Webb [24] and Massie [25] with results that may be regarded as indefinite, and the flavine compounds have been employed by a number authors, Drummond and McNee,[26] Fleming,[27] Pearson [28] Carslaw and Templeton [29] and Ligat.[30] They apparently penetrate to some extent and at least inhibit bacterial growth. They are said to produce rapid granulation.

There is little evidence that any of the dyestuffs have been used successfully in the treatment of closed cavities, except in the work of Churchman,[31] who treated joint infections by means of lavage with gentian violet solution. Better results in this group of infections, particularly in empyema and in the treatment of carriers, have apparently been reached by the use of hypochlorite solutions. A discussion of this aspect of the problem, however, does not enter within the field we are considering.

Our own results show that the streptococcus is readily destroyed under artificial conditions in the test tube by a large group of dyestuffs, much more readily than the typhoid bacillus and probably also the staphylococcus. Among the most active of these substances are certain dyes of the triphenylmethane series and the acridine compounds. When these dyestuffs are tested for their action on streptococci in the pus of experimental empyema, many of them lose a great part or all of their bactericidal properties. A few dyes, however, have been found which were as active in pus as in broth, among which may be mentioned safranin, methyl green, basic fuchsin, acriflavine and proflavine.

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[22] Jour. Roy. Army Med. Corps, 1917, 29, p. 560.

[23] Jour. Am. Med. Assn., 1920, 74, p. 145.

[24] Jour. Roy. Army Med. Corps., 1918, 31, p. 315.

[25] Lancet, 1918, 194, p. 635.

[26] Lancet, 1917, 193, p. 640.

[27] Lancet, 1917, 193, p. 341.

[28] Lancet, 1918, 194, p. 370.

[29] Lancet, 1918, 194, p. 634.

[30] Brit. Med. Jour., 1917, 1, p. 78.

[31] Jour. Am. Med. Assn., 1918, 70, p. 1047.

Several of the dyestuffs found to be active in pus have been tested for therapeutic effects in the experimental streptococcus empyema, which may be produced under proper conditions with uniformity by direct inoculation into rabbits. In no instance was a distinct curative effect produced, owing to various reasons, some of which have been analyzed, particularly in respect to the most favorable of these dyestuffs—acriflavine. Although many of these dyes are more or less toxic for the animal, they can be utilized for direct injection into the pleural cavity in a dosage which should theoretically sterilize. The best of these—acriflavine—given in a dosage many times more concentrated than the bactericidal titer in the test tube, does not lose its color in the animal body for some time, and kills a good many organisms. The pleural fluids often become practically sterile, but apparently become reinfected. There is no evidence that the streptococcus becomes dye fast to acriflavine, but it may be protected in the masses of fibrin that line the pleural cavity. Phagocytosis is inhibited in strong concentrations of acriflavine, such as have usually been employed, but the dye will sterilize in the test tube considerable quantities of pus in a dose which does not inhibit the phagocytic activity of the leukocytes. The failure of acriflavine to act successfully in the pleural cavity is not due to the failure of oxygen. As suggestive results have been obtained by the use of immune serum, combinations of immune serum and acriflavine have been utilized also, but without success in sterilizing the pleural cavity or in prolonging the life of the animal. In a number of experiments repeated treatments with and without the aspiration of the accumulated fluid have also failed.

Our results do not justify anticipation of practical therapeutic results from the use of dyestuffs in empyema due to the streptococcus. It should be added, however, that we have not as yet considered the possibilities of using dyestuffs combined with metallic compounds, such as those with mercury described by Davis, White and Rosen[32] and by Young, White and Swartz.[33]

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[32] Jour. Urology, 1918, 2, p. 277.

[33] Jour. Am. Med. Assn., 1919, 73, p. 1483.